A New Class of Highly Efficient, Non-peptidic Oligoguanidinium Vectors that Selectively Internalize into Mitochondria

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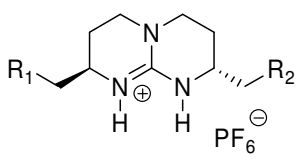
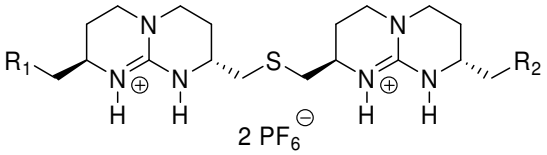
Supporting Information

General. Fmoc-protected amino acids were purchased from Advanced Chem Tech. Coupling reagents: 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) was purchased from Applied Biosystems; Rink amide MBHA resin was purchased from NovaBiochem; 1-hydroxy-7-azabenzotriazole (HOAt) was purchased from GL Biochem; 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (HOBT) were purchased from Albatros Chem Inc. Solvents: trifluoroacetic acid (TFA), piperidine, dimethylformamide (DMF), dichloromethane, acetonitrile, methanol and diethyl ether were purchased from SDS. Reagents: potassium thioacetate, ammonium hexafluorophosphate, methanesulfonic anhydride, tributylphosphine, cesium carbonate and cystamine were purchased from Aldrich; methanesulfonic acid, *N*-methyl morpholine (NMM) and 5(6)-carboxyfluorescein (CF) were purchased from Acros; benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from NovaBiochem; diisopropylcarbodiimide (DIC) and N,N-diisopropylethylamine (DIEA) were purchased from Merck; triisopropylsilane (TIS) and potassium hexafluorophosphate were purchased from Fluka. Column chromatography was performed using silica gel 60Å (Scharlau, 40-60 μm) and reverse-phase silica gel LiChroprep® RP-18 (Merck, 25-40 μm). Thin layer chromatography (TLC) was performed on Alugram Sil G/UV254-coated aluminium sheets (Macherey-Nagel) with detection by UV at 254/365 nm and/or with bromocresol green (1.4 mM in EtOH, 4% 0.1 M NaOH). Melting points were determined on a Gallenkamp apparatus. Optical rotations $[\alpha]^{20}_{D}$ were determined on a Perkin-Elmer 241 MC polarimeter, using a quartz cell (1 dm) at 298 K (Na_D 589 nm). ^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX-300 and DRX-500 spectrometers at 298 K. Chemical shifts (δ) are expressed in ppm relative to the solvent residual peak. ^{13}C NMR spectra were assigned using DEPT (distortionless enhancement by polarization transfer) experiments. Mass spectra by fast atom bombardment (FAB) were recorded on a VG AutoSpec spectrometer using *m*-nitrobenzyl alcohol as matrix and by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) method on a REFLEX spectrometer using ditranol as matrix. Elemental analyses were performed on a LECO CHNS 932 microanalyser.

Synthesis.



7 R_1 = OTBDPS; R_2 = OMs



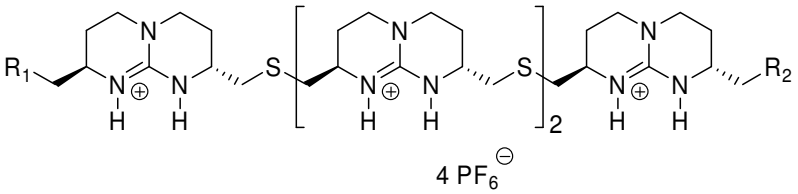
8 R_1 = OTBDPS; R_2 = OH

10 R_1 = OTBDPS; R_2 = OMs

Compound 8. A solution of mesylate **7**[1] (1.90 g, 2.87 mmol) and potassium thioacetate (0.426 g, 3.73 mmol) in a mixture of THF (40 ml) and water (15 ml) was refluxed for 24 h. After cooling to room temperature MsOH (0.930 ml, 14.35 mmol) was added and the mixture was refluxed again for 24 h. The reaction mixture was cooled to room temperature, water (150 ml) and Et_2O (150 ml) were added and after extraction both phases were separated. The organic phase was extracted once again with water (30 ml). The combined aqueous phases were washed with $CHCl_3$ (150 ml) and Et_2O (150 ml). After concentration of about 30% of the aqueous layer, $KHCO_3$ (1.72 g, 17.22 mmol) was added and the solvent evaporated to dryness. Then MeOH (100 ml) was added, the precipitate was removed by filtration and the solvent evaporated. This procedure was repeated a few times with increasing amounts of CH_2Cl_2, until pure CH_2Cl_2 (20 ml), resulting in a slightly yellow solid (0.93 g). To a solution of this product and Cs_2CO_3 (840 mg, 2.58 mmol) in MeOH (30 ml) tributylphosphine (415 µl, 1.55 mmol) was added and the mixture was stirred for 40 min at room temperature. Then a solution of mesylate **7** (1.71 g, 2.58 mmol) in THF (50 ml) was added and the mixture was stirred for 1 h at the same temperature. After evaporation of the solvent, CH_2Cl_2 and 0.1 M aq NH_4PF_6 were added. After phase separation the aqueous layer was extracted once more with CH_2Cl_2 and the combined organic phases were filtered over cotton and concentrated to dryness. The crude residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 4% → 6%), yielding **8** (1.99 g, 75%) as dihexafluorophosphate salt. mp 101°C; $[\alpha]_D^{20}$ -96 (*c* = 0.4, MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.67-7.60 (m, 4H), 7.45-7.35 (m, 6H), 6.48 (br s, 1H), 6.33 (m, 2H), 6.22 (br s, 1H), 3.73-3.20 (m, 16H), 2.90-2.45 (m, 4H), 2.15-1.30 (m, 8H), 1.08 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz, DEPT) δ 150.7 (C), 150.5 (C), 135.4 (CH), 132.7 (C), 132.5 (C), 129.9 (CH), 127.8 (CH), 65.3 (CH_2), 64.4 (CH_2), 50.2 (CH), 49.8 (CH), 47.6 (CH), 47.1 (CH), 45.3 (CH_2), 44.8 (CH_2), 36.0 (CH_2), 35.7 (CH_2), 26.7 (CH_3), 26.5 (CH_2), 25.7 (CH_2), 22.4 (CH_2), 22.2 (CH_2), 19.0 (C); HRMS (FAB$^+$) *m/z* 635.3574 (calcd 635.3563) $[M - HPF_6 - PF_6]^+$; MS (FAB$^+$) *m/z* 781.2 (15) $[M - PF_6]^+$, 635.2 (100) $[M - HPF_6 - PF_6]^+$; elemental analysis calcd (%) for $C_{34}H_{52}F_{12}N_6O_2P_2SSi$: C 44.06, H 5.65, N 9.07, S 3.46; found: C 44.85, H 5.33, N 8.85, S 3.89.

Compound 10. To a solution of alcohol **8** (0.270 g, 0.291 mmol) and NMM (96 µl, 0.874 mmol) in THF (8 ml) at 0°C was added a solution of Ms_2O (0.105 g, 0.439 mmol) in THF (2 ml) and the

mixture was stirred for 2 h at this temperature. The solvent was evaporated, CH_2Cl_2 (40 ml) and 0.1 M aq NH_4PF_6 (20 ml) were added and, after extraction, the organic phase was separated. The aqueous phase was extracted once more with CH_2Cl_2 (10 ml). The combined organic layers were filtered over cotton, concentrated to dryness and the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 5%), affording **10** (276 mg, 94%) as dihexafluorophosphate salt. mp 78°C; $[\alpha]_D^{20}$ -83 (c = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.63-7.65 (m, 4H, Ar), 7.39-7.45 (m, 6H, Ar), 6.32 (s, 1H, NH), 6.26 (s, 1H, NH), 6.08 (s, 1H, NH), 4.21-4.24 (AB-system, J_{AB} = 10.6 Hz, 1H, CH_2O), 4.07-4.10 (AB-system, J_{AB} = 10.6 Hz, 1H, CH_2O), 3.72-3.75 (m, 1H), 3.64-3.68 (AB-system, J_{AB} = 10.4 Hz, 1H, CH_2O), 3.59-3.61 (m, 4H), 3.33-3.44 (m, 4H), 3.24-3.40 (m, 1H), 3.02 (s, 3H, CH_3SO_2), 2.82-2.88 (m, 2H, CH_2S), 2.66-2.72 (m, 2H, CH_2S), 2.05-2.14 (m, 4H, $CH_2\beta$), 1.87-2.04 (m, 4H, $CH_2\beta$), 1.06 [s, 9H, $C(CH_3)_3$]; ^{13}C NMR ($CDCl_3$, 125 MHz, DEPT) δ 151.3 (C), 151.2 (C), 135.6 (2 × CH), 132.8 (CH), 130.0 (CH), 127.9 (CH), 69.1 (CH_2), 65.5 (CH_2), 49.7 (CH), 47.4 (CH), 47.2 (CH), 46.4 (CH_2), 45.4 (CH_2), 45.1 (CH_2), 45.0 (CH_2), 44.8 (CH_2), 37.3 (CH_2), 37.0 (CH_3), 36.9 (CH_2), 26.8 (CH_2), 26.1 (CH_2), 22.6 (CH_3), 22.2 (CH_3), 19.2 (C); MS (FAB$^+$) *m/z* 713.5 $[M - HPF_6 - PF_6]^+$, 859.3 $[M - PF_6]^+$; elemental analysis calcd (%) for $C_{35}H_{54}F_{12}N_6O_4P_2S_2$: C 41.83, H 5.42, N 8.36, S 6.38; found: C 41.92, H 5.33, N 8.22, S 6.34.



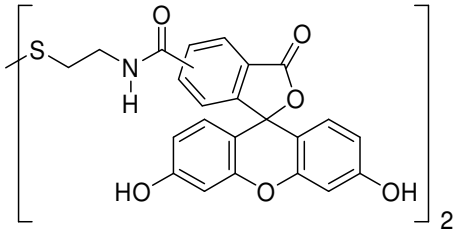
11 R_1 = OTBDPS; R_2 = OH

12 R_1 = OTBDPS; R_2 = OMs

Compound 11. A solution of mesylate **10** (640 mg, 0.637 mmol) and potassium thioacetate (370 mg, 3.18 mmol) in a mixture of THF (25 ml) and water (10 ml) was refluxed for 18 h. After cooling to room temperature MsOH (0.62 ml, 9.55 mmol) was added and the mixture was refluxed for 24 h. The reaction was cooled to room temperature, water (150 ml) and Et_2O (150 ml) were added and, after extraction, the phases were separated. The aqueous phase was washed with $CHCl_3$ (150 ml) and once again with Et_2O (150 ml). After evaporation of about 50% of the aqueous layer, $KHCO_3$ (1.1 g, 10.8 mmol) was added and the solvent was evaporated to dryness. Then MeOH (100 ml) and CH_2Cl_2 (20 ml) were added, the precipitate was removed by filtration and the solvent was evaporated. This procedure was repeated a few times with increasing amounts of CH_2Cl_2, until pure CH_2Cl_2 (150 ml), resulting in a slightly yellow solid (352 mg). To a solution of this product and Cs_2CO_3 (170 mg, 0.50 mmol) in MeOH (12 ml) tributylphosphine (80 µl, 0.30 mmol) was added

and the reaction was stirred for 30 min at room temperature. Then a solution of mesylate **10** (503 mg, 0.50 mmol) in THF (12 ml) was added and the mixture was stirred for 1 h. After evaporation of the solvent, CH_2Cl_2 (70 ml) and 0.1 M aq NH_4PF_6 (50 ml) were added. After phases separation, the aqueous layer was extracted with CH_2Cl_2 (2 × 20 ml) and the combined organic phases were filtered over cotton and concentrated to dryness. The crude residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 3% → 5%), yielding **11** (625 mg, 61%) as tetrahexafluorophosphate salt. mp 88°C; $[\alpha]_D^{20}$ -132 (*c* = 0.5, MeOH); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.73-7.70 (m, 4H), 7.53-7.45 (m, 6H), 7.32 (m, 4H), 7.29 (br s, 1H), 7.26 (br s, 1H), 7.15 (br s, 1H), 7.11 (br s, 1H), 3.85-3.65 (m, 10H), 3.62-3.48 (m, 18H), 3.07-2.98 (m, 6H), 2.78-2.68 (m, 6H), 2.28-1.80 (m, 16H), 1.08 (s, 9H); ^{13}C NMR (acetone-d_6, 125 MHz, DEPT) δ 151.3 (2 × C), 151.2 (C), 135.7 (2 × CH), 133.2 (C), 133.1 (C), 130.3 (2 × CH), 128.2 (2 × CH), 66.3 (CH_2), 64.4 (CH_2), 51.1 (CH), 50.6 (CH), 48.2 (CH), 48.1 (CH), 48.0 (CH), 45.7 (CH_2), 45.5 (CH_2), 45.4 (CH_2), 45.3 (CH_2), 36.3 (CH_2), 36.2 (CH_2), 36.1 (CH_2), 26.6 (CH_3), 26.0 (CH_2), 25.8 (CH_2), 25.7 (CH_2), 22.8 (CH_2), 22.7 (CH_2), 19.15 (C); HRMS (FAB$^+$) *m/z* 1029.5512 (calcd 1029.5537) $[M - HPF_6 - PF_6]^+$; MS (FAB$^+$) *m/z* 1467.5 (92) $[M - PF_6]^+$, 1321.7 (100) $[M - HPF_6 - PF_6]^+$, 1175.5 (78) $[M - 2HPF_6 - PF_6]^+$, 1029.5 (76) $[M - 3HPF_6 - PF_6]^+$; elemental analysis calcd (%) for $C_{52}H_{84}F_{24}N_{12}O_2P_4S_3Si$: C 38.71, H 5.19, N 10.42, S 5.96; found: C 39.14, H 4.89, N 10.28, S 6.50.

Compound 12. To a solution of alcohol **11** (107 mg, 0.066 mmol) and NMM (58 µl, 0.52 mmol) in CH_3CN (2 ml) was added a solution of Ms_2O (105 mg, 0.20 mmol) in CH_3CN (1 ml) and the mixture was stirred for 2 h at room temperature. The solvent was evaporated, CH_2Cl_2 and 0.1 M aq NH_4PF_6 were added and, after extraction, the organic phase was separated. The aqueous phase was extracted once more with CH_2Cl_2. The combined organic layers were filtered over cotton and concentrated to dryness and the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 3% → 5%), affording **12** (107 mg, 95%) as tetrahexafluorophosphate salt. mp 111°C; $[\alpha]_D^{20}$ -117 (*c* = 0.4, MeOH); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.73-7.70 (m, 4H), 7.53-7.45 (m, 6H), 7.40 (br s, 1H), 7.20 (m, 6H), 7.02 (br s, 1H), 4.47 (dd, *J* = 4.1, 10.5 Hz, 1H), 4.29 (dd, *J* = 7.5, 10.5 Hz, 1H), 4.00 (m, 1H), 3.85-3.70 (m, 9H), 3.61-3.48 (m, 16H), 3.20 (s, 3H), 3.07-2.98 (m, 6H), 2.78-2.69 (m, 6H), 2.30-2.18 (m, 8H), 2.04-1.90 (m, 8H), 1.08 (s, 9H); ^{13}C NMR (acetone-d_6, 125 MHz, DEPT) δ 151.9 (C), 151.8 (2 × C), 136.3 (CH), 136.2 (CH), 133.7 (C), 133.6 (C), 130.9 (2 × CH), 128.8 (2 × CH), 71.7 (CH_2), 66.9 (CH_2), 51.2 (CH), 48.7 (CH), 48.6 (CH), 46.0 (CH_2), 45.9 (CH_2), 45.5 (CH_2), 37.2 (CH), 36.6 (CH_2), 27.2 (CH_3), 26.3 (CH_2), 26.2 (CH_2), 23.2 (CH_2), 22.6 (CH_2), 19.7 (C); HRMS (FAB$^+$) *m/z* 1545.4408 (calcd 1545.4472) $[M - PF_6]^+$; MS (FAB$^+$) *m/z* 1545.8 (100) $[M - PF_6]^+$, 1399.6 (70) $[M - HPF_6 - PF_6]^+$, 1253.7 (30) $[M - 2HPF_6 - PF_6]^+$, 1107.5 (6) $[M - 3HPF_6 - PF_6]^+$.



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Compound 13. A mixture of 5(6)-carboxyfluorescein (575 mg, 1.528 mmol), cystamine (0.172 g, 0.76 mmol), pyBOP (0.875 g, 1.68 mmol), DIEA (1.2 ml, 6.88 mmol) and HOBT (catalytic amount) in DMF (30 ml) was stirred overnight at room temperature. The solvent was evaporated, Et_2O was added and, after sonication, the organic layer was removed. Then $CHCl_3$ was added to precipitate the product, which was filtered off and purified by column chromatography on silica gel (CH_2Cl_2/MeOH/AcOH, 92:8:2), resulting in **13** (330 mg, 50%).

General Procedure for the Syntheses of CF-RKKRRQRRR-NH_2 (CF-Tat) and CF-RQIKIWFQNRRMKWKK-NH_2 (CF-Antp). The syntheses of H_2N-RKKRRQRRR-Rink Amide MBHA resin and H_2N-RQIKIWFQNRRMKWKK-Rink Amide MBHA resin were carried out automatically on an Applied Biosystems 433A Peptide Synthesizer. Rink Amide MBHA resin (0.1 mmol, 128 mg; Initial loading of the resin = 0.78 mmol/g), 10 eq of amino acid and a solution of 0.45 M TBTU/HOBT and 2 M DIEA in N-methylpyrrolidine (NMP) were used for coupling reactions. After completion of the sequences, H_2N-R(Pbf)K(Boc)K(Boc)R(Pbf)R(Pbf)Q(Trt)R(Pbf)R(Pbf)R(Pbf)-Rink Amide MBHA and H_2N-R(Pbf)Q(Trt)IK(Boc)IW(Boc)FQ(Trt)N(Trt)R(Pbf)R(Pbf)MK(Boc)W(Boc)K(Boc)K(Boc)-Rink Amide MBHA, peptide-resins were treated with a solution of CF (5 eq), PyAOP (5 eq), HOAt (5 eq) and DIEA (10 eq) dissolved in DMF/CH_2Cl_2 9/1, preactivated for 10 min before addition, and stirred for 1.5 h. The labeled peptides were cleaved from the resin by treatment with 81.5% TFA, 5% thioanisole, 5% water, 5% EDT, 1% TIS, 5% phenol for 4 h. The labeled peptides were identified at λ = 443 nm by analytical RP-HPLC [Waters 996 photodiode array detector equipped with the Waters 2695 separation module, Symmetry column (C18, 5 μm, 4.6 × 150 mm) and Millennium software; Flow = 1 ml/min; Gradient = 5-100% B in 15 min (B = 0.036% TFA in acetonitrile)]. CF-peptides were purified by semi-preparative RP-HPLC [Waters 2487 Dual λ Absorbance Detector equipped with the Waters 2700 Sample Manager, Waters 600 Controller, Waters Fraction Collector, Symmetry® column (C18, 5 μm, 30 × 100 mm) and Millennium chromatography manager software; Flow = 10 ml/min; Gradient = 5-20% D in 5 min; 20-70% D in 30 min; 70-100% D in 5 min (D = 0.1% TFA in acetonitrile)] and further characterized by MALDI-TOF MS (Vogayer-DE RP MALDI-TOF, PE Biosystems with a N_2 laser of 337 nm).

CF-RKKRRQRRR-NH_2: *m/z* 1698 (Calcd 1697) $[M + H]^+$, 1720 $[M + Na]^+$.

CF-RQIKIWFQNRRMKWKK-NH_2: *m/z* 1303 (Calcd 2604) $[M + 2H]^{2+}$, 869 $[M + 3H]^{3+}$, 652 $[M + 4H]^{4+}$, 522 $[M + 5H]^{5+}$.

Combination of experimental absorption values of the different CF-peptides obtained by UV at 490 and 443 nm and Amino Acid Analysis allowed the exact concentration to be determined for each sample.

MTT assay. For each assay 7×10^3 cells/cm^2 were seeded in a 96-well plate (Nalge Nunc International) and cultured for 24 h. Compounds were added at concentrations ranging from 1 μM to 50 μM. Cells were incubated for 24 h at 37ºC under a 5% CO_2 atmosphere. After 22 h, MTT was added at 0.5 mg/ml final concentration. The cells with peptide and MTT were incubated for a further 2 h and then the medium was discarded. Isopropanol was added to dissolve the formazan product and 30 min later absorbance was measured at $\lambda = 570$ nm. The cell viability is expressed as a percentage ratio of cells treated with the different compounds against untreated cells.

References

1. Breccia, P., Van Gool, M., Perez-Fernandez, R., Martin-Santamaria, S., Gago, F., Prados, P. & de Mendoza, J *J. Am. Chem. Soc.* **2003**, *125*, 8270−8284.